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Vaccine 24 (2006) 1501–1514
Vaccine
www.elsevier.com/locate/vaccine

A recombinant 63-kDa form of *Bacillus anthracis* protective antigen produced in the yeast *Saccharomyces cerevisiae* provides protection in rabbit and primate inhalational challenge models of anthrax infection

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Received 15 July 2005; received in revised form 5 October 2005; accepted 10 October 2005
Available online 21 October 2005

Abstract

Infection by *Bacillus anthracis* is preventable by prophylactic vaccination with several naturally derived and recombinant vaccine preparations. Existing data suggests that protection is mediated by antibodies directed against the protective antigen (PA) component of the anthrax toxin complex. PA is an 83-kDa protein cleaved in vivo to yield a biologically active 63-kDa protein. In an effort to evaluate the potential of yeast as an expression system for the production of recombinant PA, and to determine if the yeast-purified rPA63 can protect from a lethal inhalational challenge, the sequence of the 63-kDa form of PA was codon-optimized and expressed in the yeast *Saccharomyces cerevisiae*. Highly purified rPA63 isolated from *Saccharomyces* under denaturing conditions demonstrated reduced biological activity in a macrophage-killing assay compared to non-denatured rPA83 purified from *Escherichia coli*. Rabbits and non-human primates (NHP) immunized with rPA63 and later challenged with a lethal dose of *B. anthracis* spores were generally protected from infection. These results indicate that epitopes present in the 63-kDa from of PA can protect rabbits and non-human primates from a lethal spore challenge, and further suggest that a fully functional rPA63 is not required in order to provide these epitopes.

Keywords: Anthrax; Vaccine; Protective antigen

1. Introduction

Anthrax, a disease caused by the spore-forming bacterium *Bacillus anthracis*, is highly lethal in the inhalational form (for general reviews see [1–3]). Infection occurs when the *Bacillus* spores are inhaled by the host and taken up by local macrophages in the lung where they germinate into vegetative bacilli. These cells secrete three proteins known as lethal factor (LF), edema factor (EF), and protective antigen (PA). During infection, the 83-kDa PA is cleaved after the sequence Arg-Lys-Lys-Arg at amino acid position 167 by a mammalian furin-like protease to yield an active 63-kDa component that spontaneously oligomerizes and combines with LF and EF to form the highly lethal anthrax toxin [4–6].

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0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.10.018

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
6 MAR 2005	**N/A**	**-**

4. TITLE AND SUBTITLE
A recombinant 63-kDa form of Bacillus anthracis protective antigen produced in the yeast Saccharomyces cerevisiae provides protection in rabbit and primate inhalational challenge models of anthrax infection. Vaccine 24:1501 - 1514

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hepler, RW Kelly, R McNeely, TB Fan, H Losada, MC George, HA Woods, A Cope, LD Bansal, A Cook, JC Zang, G Wei, X Cohen, SL Keller, PM Leffel, E Joyce, JG Pitt, L Schultz, LD Jansen, KU Kurtz, M

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Institute of Infectious Diseases, Fort Detrick, MD

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Infection by Bacillus anthracis is preventable by prophylactic vaccination with several naturally derived and recombinant vaccine preparations. Existing data suggests that protection is mediated by antibodies directed against the protective antigen (PA) component of the anthrax toxin complex. PA is an 83-kDa protein cleaved in vivo to yield a biologically active 63-kDa protein. In an effort to evaluate the potential of yeast as an expression system for the production of recombinant PA, and to determine if the yeast-purified rPA63 can protect from a lethal inhalational challenge, the sequence of the 63-kDa form of PA was codon-optimized and expressed in the yeast Saccharomyces cerevisiae. Highly purified rPA63 isolated from Saccharomyces under denaturing conditions demonstrated reduced biological activity in a macrophage-killing assay compared to non-denatured rPA83 purified from Escherichia coli. Rabbits and non-human primates (NHP) immunized with rPA63 and later challenged with a lethal dose of B. anthracis spores were generally protected from infection. These results indicate that epitopes present in the 63-kDa from of PA can protect rabbits and non-human primates from a lethal spore challenge, and further suggest that a fully functional rPA63 is not required in order to provide these epitopes.

15. SUBJECT TERMS
Bacillus anthracis, anthrax, protective antigen, PA, recombinant, yeast expression, laboratory animals, rabbits, nonhuman primates

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**14**	

With an increased awareness of the potential of *B. anthracis* as a bioterrorist weapon, the need for a safe, effective, and readily available vaccine for wide public use has grown [7]. Vaccine preparations for human use have traditionally been attenuated live spores or cell-free secretion products of *B. anthracis* adsorbed to aluminum adjuvants [8,9]. The former preparation was used in the Soviet Union and was associated with a number of toxicities and production issues [10]. In the US, the latter is used exclusively and is currently sold as Biothrax™. Biothrax™ is a sterile culture filtrate from an attenuated non-encapsulated strain of *B. anthracis* adsorbed to an aluminum adjuvant, which contains unknown amounts of the three toxin components [10]. This vaccine requires a rigorous immunization schedule consisting of six doses over 18 months with an additional annual booster thereafter. Neither vaccine has the characteristics preferred for broad distribution in the event of an emergency, or for general public prophylaxis, i.e., long-term protection, high-level efficacy with a limited number of immunizations, low reactogenicity, and a reliable, reproducible means of production.

Although there are limited data in humans correlating protection against *B. anthracis* infection to specific immune responses, the available evidence suggests that antibodies directed against the 83-kDa form of PA (PA83) is a surrogate marker for protective immunity [11,12]. The data are more extensive in animal protection models using guinea pigs, rabbits, mice, and rhesus monkeys [13–15]. Immunization with purified native or recombinant PA83 confers protection against challenge in rhesus macaques [13], and passive protection was demonstrated in guinea pigs, mice, and rabbits with anti-PA83 antibodies [16–18]. There is a rough correlation between anti-PA83 titer and protection against lethal challenge with *B. anthracis* spores. Reuveny et al. [19] reported a more quantitative correlate with neutralization of in vitro macrophage killing for the guinea pig model. The cumulative evidence supports the concept that PA83, whether obtained from *B. anthracis*, or as a recombinant protein, can be an effective immunogen for anthrax prophylaxis.

Anthrax treatment and/or prophylaxis also show promise with mAbs to PA. In one report, mAbs to PA were generated by engrafting human peripheral blood lymphocytes from anthrax-vaccinated donors into severe combined immunodeficient (SCID) mice, followed by hybridoma formation and rescue of the genes encoding the protective antibodies [20]. Stable cell lines expressing full-length human immunoglobulin were subsequently created. These mABs protected rats from a lethal dose of anthrax toxin in the Fisher 344 rat bolus toxin challenge model. More recently, a phase I clinical study was reported with PAmAb, a fully humanized mAb against PA that had demonstrated protection against challenge with *B. anthracis* spores in rabbit and monkey models of inhalational anthrax [21]. The mAb was safely administered to more than 100 healthy volunteers and shown to be well tolerated and bioavailable after either a single intravenous or intramuscular dose.

Published data show that the protective epitopes of PA83 can be found within the 63-kDa form of the protein [1,22–24]. There is some evidence that PA63 is also a protective immunogen. Ivins and Welkos cited a personal communication noting that immunization of guinea pigs with a 65-kDa fragment of PA83, produced by trypsinization, afforded protection against a challenge with virulent spores of the Sterne strain [25]. However, a replicon construct designed to express the 63-kDa form of PA failed to protect immunized mice against challenge with spores from the Sterne strain, whereas a similar construct designed to express PA83 was protective [26]. Immunization of mice with DNA constructs designed to express either secreted or cellular PA63 (or PA83) conferred protection against challenge with anthrax toxin [27,28]. Galloway et al. showed that immunization with a PA63 construct followed by a boost with rPA83 afforded protection in a rabbit challenge model with spores of the Ames strain, but only one rabbit with this immunization regime was evaluated [29].

A variety of alternative vaccine preparations designed to minimize the occurrence of adverse experiences are currently being evaluated (for reviews see [8,9,27,30]). These include attenuated *B. anthracis* strains with enhanced PA83 production, recombinant protein products, and DNA-based vaccines. Containment issues related to using a live, attenuated strain of *B. anthracis* as a host may limit consideration of this system for large-scale production of PA, but recombinant production of toxin components appears to be a safe and viable alternative [8,9,30]. Production of rPA83 in *Escherichia coli* has been investigated extensively [30–35], and PA83 has also been expressed in transgenic tobacco chloroplasts [36]. An alternative expression system utilizing *Bacillus subtilis*, a related but non-pathogenic sporulating *Bacillus* spp. was reported to have high endogenous protease activity that resulted in PA83 degradation [12].

Saccharomyces cerevisiae (Baker's yeast) is generally recognized as an effective system for production of recombinant proteins, and antigens isolated from yeast have been shown to have good safety profiles. Examples of successful production in *S. cerevisiae* include recombinant hepatitis B surface antigen [37], and human papillomavirus virus-like particles [38,39]. In addition to safety, the *S. cerevisiae* expression system has a number of other benefits including ease of cultivation under conditions of minimal containment, and the ability to produce both small peptides and large proteins. Due to all of these features, yeast is being tested for production of a variety of additional antigens [40].

Based on the advantages of the yeast expression system for vaccine production, we wished to evaluate it for the production of recombinant PA. Here we present the design, expression, and purification of the 63-kDa activated form of PA, and demonstrate its' protective efficacy in two independent animal models of inhalational anthrax infection.

2. Material and methods

2.1. Strains and media

S. cerevisiae strain 1260 (*MATa*, *leu2*, *mnn9*, *ura3Δ*, *can1*, *his3Δ::GAL10p-GAL4-URA3*, *prb1Δ::HIS3*, cir°) was constructed using standard yeast genetic techniques. Strain 8-3 is a Leu^+ transformant of 1260 designed to produce rPA63 intracellularly. Infectious anthrax spores were prepared from the *B. anthracis* Ames strain [41]. pET22b-PA-WT was obtained from Collier and co-workers [42]. The Leu^+ yeast expression vectors pGAL110 [43] and pKH4α2 [44] were described previously.

Three different defined media lacking leucine were used for cultivation and fermentation of *S. cerevisiae*. SD-Leu medium was made from SD-Leu powder per the manufacturer's instructions (QBIOgene, Carlsbad, CA). 5X minus leucine medium was described by Bayne et al. [45]. SD5-Leu medium contains 0.85% Yeast Nitrogen Base without added amino acids or ammonium sulfate, 1.0% succinic acid, 0.5% ammonium sulfate, 0.04% adenine, 0.03% L-isoleucine and L-phenylalanine, 0.025% L-tyrosine, 0.020% uracil, L-tryptophan and L-lysine, 0.01% L-arginine, 0.005% L-methionine and L-histidine, adjusted to pH 5.3 with sodium hydroxide. YEHDG is a complex medium and is comprised of YEHD [46] plus 4.0% (w/v) D(+) galactose. High-density medium is a chemically defined medium [47] optimized for cultivation of yeast strain 8-3. Tryptic soy agar was obtained from Difco (Franklin Lakes, NJ). Leighton and Doi medium [48] contains 16 g/L nutrient broth, 0.5 g/L magnesium sulfate, 2 g/L potassium chloride, 1 mM calcium nitrate, 0.1 mM manganese chloride, 1 μM ferric sulfate, and 0.1% glucose.

2.2. ELISA

rPA63 was quantitated in selected samples by sandwich ELISA utilizing monoclonal antibodies (MAb) to PA83 for capture and detection (#C86301M and #C86613M, respectively, Biodesign International, Saco, ME). #C86301M was used at a final concentration of 5 μg/mL. C86613M was biotinylated with EZ-link™ Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer's directions and used at a concentration of 1 μg/mL. Antibody binding was measured by dissociation-enhanced time-resolved fluorometry with europium streptavidin (DELFIA Eu-N1 labeled streptavidin, Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer. Non-specific binding was determined by subtracting the value obtained with a cell lysate prepared from a vector-containing transformant, from that of an rPA63-expressing transformant. The amount of rPA63 was determined from a standard curve of rPA83 purified from *E. coli*. The sensitivity of detection of rPA83 was 10 ng/mL.

Sera were assessed for anti-PA antibody titers by direct ELISA. Ninety-six-well plates were coated with 100 ng/well of *E. coli*-expressed rPA83. Sera were serially diluted and transferred to the coated plates. Bound primary antibody was detected using an appropriate HRP-conjugated secondary antibody. Plates were developed using 3,3′,5,5′ tetramethylbenzidine (TMB) and end-point titers were determined using a cut-off of three times background.

2.3. SDS-PAGE and Western blotting

SDS-PAGE of process stream samples and final product was performed using either 10–20% Tris–HCl Criterion gels in 1X Tris glycine SDS buffer (Bio-Rad, Hercules, CA) or 4–20% NuPAGE gels with MOPS running buffer (Invitrogen, Carlsbad, CA). PVDF or nitrocellulose membranes were used for Western blots. Dot-blots were performed by diluting samples in 25 mM Tris, 0.15 M NaCl, pH 8 (TBS) containing 0.05% Tween 20 and applying to a nitrocellulose membrane using a Bio-Rad Biodot apparatus following the manufacturer's directions. MAb C86613M was used as the primary antibody and goat anti-mouse-IgG horseradish peroxidase-linked whole antibody (Zymed Laboratories, South San Francisco, CA) was the secondary antibody. Blots were developed using the "WESTERN LIGHTNING™" Chemiluminesence Reagent kit (Perkin-Elmer Life Sciences). Imaging was performed using X-ray film or a KODAK Imagestation 2000. Densitometry was performed on selected gels using KODAK 1D Image Analysis Software (version 3.6).

2.4. Purification of rPA83 expressed in E. coli

Recombinant PA83 purified from *E. coli* was used as a control or standard in various studies. The protein was expressed from pET22b-PA-WT in *E. coli* BL21 and purified as described previously with minor modifications [33].

Enzyme-generated PA63 (tPA63) was derived from purified *E. coli* rPA83 by treatment with trypsin as described by Blaustein et al. [49] using trypsin (type XI) and soybean trypsin inhibitor (Sigma Chemical CO., St. Louis, MO). For certain studies, tPA63 was purified by anion exchange chromatography on a Source Q column using a 0–1 M gradient of NaCl in 20 mM HEPPS, pH 8.2.

2.5. Construction and cloning of a yeast codon-optimized PA63 sequence

The gene fragment encoding rPA63 was prepared using PCR in a step-wise fashion by the annealing and extension of 24 synthetic alternating and overlapping sense and antisense oligomers (91–110 bp in length) designed to encode the final desired sequence (the DNA sequence of the yeast codon-optimized rPA63 was deposited in GenBank and assigned accession number DQ004463). Native Pfu polymerase (Stratagene) was used for the PCR in a "touch down" strategy as follows: 95 °C, 90 s, one cycle; 95 °C, 30 s, 55 °C, 30 s, 68 °C, 75 s for 5 cycles, immediately followed by a second series of reactions; 95 °C, 30 s, 52 °C, 30 s, 68° C, 75 s for 15 cycles. The reaction was completed by incubation at 68° C

for 7 min. The final PCR used distal sense and antisense primers, 5′GTCACGGATCCTGTCTTTGGATAAGAGATCCACCTCCG3′ and 5′CGCGGATCCATTACCAATTTCGTAACCCTTCT3′, respectively, which incorporated *Bam*HI sites (underlined). The sense primer included additional upstream sequences to permit in frame cloning with the yeast alpha factor (MFα1) pre-pro secretory leader (in vector pKH4α2), and encoded the last seven amino acids of the pro-leader including its Lys-Arg *KEX2* cleavage site. The resulting 1.7-kb product was gel-isolated and cloned into pCR-Blunt II-TOPO (Invitrogen) and then subcloned into the *Bam*HI site of pGEM3zf+ (Promega, Madison, WI). Sequence errors were corrected sequentially by site-directed mutagenesis using the Quik-Change Site-directed Mutagenesis Kit (Stratagene) per the manufacturer's recommendations. A final PCR with High Fidelity PCR Supermix (Invitrogen) using an antisense primer (5′CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA3′), which incorporated a stop codon upstream of a *Bam*HI site, and a sense primer which contained a *Bam*HI site (5′-GTCACGG ATC CTG TCT TTG GAT AAG AGA-3′) amplified the corrected sequence (*Bam*HI sites are underlined). The reaction product was subcloned into a TA cloning vector (Invitrogen) to construct pMK1.

For secretion of rPA63, the *Bam*HI fragment from pMK1 was subcloned downstream of the *GAL10* promoter into the *Bam*HI site of yeast secretory vector pKH4α2. The resultant plasmid, pAGT1, contained an in frame fusion of the coding sequence of rPA63 to the pre-pro-alpha factor leader sequence. To construct a plasmid for intracellular expression, the DNA encoding the rPA63 protein carried in pMK1 was amplified with a sense primer that introduced a non-translated leader sequence and ATG codon, and an antisense primer containing TAA as stop codon. The primers were 5′CGCGGATCCCACAAAACAAA ATG TCCACCTCCGCTGGTCCAACTGTTCC3′ and 5′CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA3′, respectively, and both incorporate *Bam*HI sites (underlined). The *Bam*HI fragment was cloned into the *Bam*HI site of pGAL110 under control of the *S. cerevisiae GAL1* promoter to construct pPA63GAL110.

2.6. Cultivation of yeast transformants

Leu^+ transformants containing rPA63 were obtained with pPA63GAL110 and pAGT1 by a spheroplasting protocol [50] and selected on SD-Leu agar medium containing 1 M sorbitol. Small-scale trial cultivations of multiple yeast transformants were conducted in 5.0 mL of broth in culture tubes. Cultures were incubated at 30 °C in SD5-Leu medium containing 4.0% glucose and 0.1 M sorbitol for 18–24 h to an OD_{600} of 1.5–3.0/mL. A 0.3 mL aliquot was transferred to either 5.0 mL of SD5-Leu medium containing 2% glucose and 4% galactose or YEHDG medium. The cultures were incubated with slow rotation on a cell culture roller drum for 72 h to a final OD_{600} of 5–16.0/mL. A total of 10 ODs per sample were harvested for analysis. Cell pellets were collected by centrifugation, washed once with PBS, and broken with 0.6 g glass beads (400–625 μm, Sigma Chemical CO, St. Louis, MO) in 0.3 mL lysis buffer (0.1 M sodium phosphate buffer, pH 7.2, 0.5 M sodium chloride, 1 mM PMSF). The lysate was recovered by centrifugation, the unbroken cells/beads were washed with an additional 0.3 mL of lysis buffer and the clarified supernatants were combined. The clarified lysate was assayed for protein by the method of Bradford [51] with the Bio-Rad Protein Assay using the manufacturer's instructions. Secretion of rPA63 was evaluated directly from medium supernatants collected after centrifugation to remove the yeast cells. A variety of transformants and culture conditions (including media and time dependence) were evaluated by this method. Subsequently, 50 mL cultures of several high-producing transformants were cultivated in 250 mL shake flasks at 220 rpm. The transformant providing the highest level of expression was carried forward for scale-up in a fermentor.

For fermentation at the 20 L-scale, a frozen stock of the transformant selected from the 50 mL cultures (strain 8-3), was thawed and 1.0 mL was inoculated into a 250-mL Erlenmeyer flask with 50 mL of 5X Leu-medium containing 4% glucose. The flask was incubated at 28 °C, 250 rpm on a rotary shaker. After 24 h (residual glucose 22.3 g/L), a culture volume of 11 mL was added to a 2-L flask containing 877 mL of the same medium. Again, the flask was incubated at 28 °C and agitated at 250 rpm. After 24.5 h, the contents of the 2-L flask were used to inoculate a 20-L reactor containing high-density culture medium. This medium also contained 20 g/L glucose followed by 40 g/L galactose for induction. The reactor was operated at 28 °C, 4.7 L/min, 15 psig, and 300 rpm. Under these conditions, dissolved oxygen levels were maintained at greater than 30% saturation. Culture growth was monitored by glucose consumption, optical density at 600 nm, dry cell weight, galactose utilization, and ethanol production. Cultivation continued for 90 h reaching an optical density of 35 and a dry cell weight of 19.7 g/L. The culture was harvested by hollow fiber tangential flow filtration using a 0.1 μm cut-off membrane. Permeate was discarded and the cells were concentrated to 1.35 L, diafiltered with PBS, and collected by centrifugation at 10,800 × *g*, 4 °C for 20 min. The cell pellets were stored at −70 °C until needed.

2.7. Purification of yeast rPA63

Frozen yeast cells were thawed in a cold-water bath and re-suspended in a 5× volume of cold breaking buffer: 0.2 M HEPES, pH 7.4, 2 mM $MgCl_2$, containing 0.5 mg/mL Pefabloc (Roche Applied Science, Indianapolis, IN), 1× "Complete" EDTA free protease inhibitor mix (Roche Applied Science), 3.5 μg/mL pepstatin (Roche Applied Science), 20 mM benzamidine (Sigma–Aldrich Chemicals, St. Louis, MO). Benzonase® (EMD Biosciences, San Diego, CA), 1 μL/g wet cell weight, was added and the cells were disrupted by four passes through a microfluidizer apparatus (Microfluidics,

Model 110S, Newton MA) at a pressure of 14,000 psi. The temperature of the lysate was maintained at ≤15 °C throughout the procedure, and the pH was maintained at 7.4 without adjustment. EDTA and EGTA were added to the lysate to final concentrations of 5 mM each. Insoluble material was removed by centrifugation (10,000 × *g*) for 20 min at 4 °C. The supernatant was clarified by filtration through a series of 47 mm filters (AP15, AP25, and 5 μM (Durapore White Hydrophilic Disc SVLP, Millipore)), and the clarified lysate was divided into aliquots for storage at −70 °C.

Frozen clarified lysate (320 mL) was thawed and adjusted to 6 M urea by addition of solid reagent (Pierce Biotechnology, Rockford, IL) and all chromatographic steps were performed at 2–8 °C. Denatured lysate was applied to a 1 L POROS 50HS (Amersham Biosciences, Piscataway NJ) cation exchange column equilibrated in 20 mM HEPES, pH 7.3 containing 6 M urea at a flow rate of 35 mL/min using a Waters 650 Prep system with a Linear UV–vis detector operated at 280 nm. The column was washed with buffer until the signal returned to baseline. Elution was accomplished using a gradient of 0–1 M NaCl in equilibration buffer. Total protein in column fractions was determined using a commercial bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), and fractions were analyzed by dot-blot, SDS-PAGE, and Western blots.

rPA63-containing fractions were pooled and concentrated over a 10-kDa cut-off tangential flow membrane (Millipore PrepScale TFF). The concentrated cation exchange pool was diluted 5-fold with 6 M urea in 20 mM HEPES buffer (pH 7.3) and applied over two runs to a 50 mL Source Q (Amersham Biosciences, Piscataway, NJ) anion exchange column equilibrated in 20 mM HEPES, pH 7.3 containing 6 M urea at a flow rate of 20 mL/min. Elution was achieved using a gradient of 0–1 M NaCl in equilibration buffer and rPA63-containing fractions were pooled and dialyzed extensively against 20 mM HEPES, pH 7.3 containing 0.15 M NaCl. Sixteen micromolar $CaCl_2$ was added to the refolding buffer based on studies showing a role for calcium in the folding of PA [52]. The anion exchange pool was sterile-filtered at 0.2 μm and stored in aliquots at −70 °C.

2.8. Biochemical characterization

The fluorescent dye 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid (TNS) [53] was used to assess the relative hydrophobicity of the various purified forms of PA. Concentrated samples of yeast rPA63 or tPA63 were diluted into a stock solution of 50 μg/mL TNS (Molecular Probes, Eugene OR) in 0.1 M HEPES, pH 7.3, 0.15 M sodium chloride, 1 mM EDTA. The fluorescence spectra were obtained from 370 to 500 nm using an excitation wavelength of 466 nm in a Perkin-Elmer LS 50B Luminescence Spectrometer. The spectrum for TNS alone was used as a blank [54,55].

Yeast rPA63 was analyzed by circular dichroism (CD) using a JASCO J-810 spectrapolarimeter. Purified rPA63 at 1 mg/mL in 20 mM HEPES, pH 7.3 was diluted 5-fold in 10 mM sodium phosphate, pH 7.4. Spectra were obtained at 25 °C from 260 to 185 nm and averaged over 10 scans. Quantitative amino acid analysis was used to accurately determine the protein concentration of the CD samples, and the raw data were analyzed using CDPro software [56].

Mass determination was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on either intact rPA63 or rPA63 cleaved chemically with cyanogen bromide (CNBr), or enzymatically with EndoLys-C (LysC). For intact protein and CNBr digestion analysis, an aliquot of the mixture was spotted onto target plates coated with an ultrathin layer of the matrix [57]. For analysis of Lys-C products (low or high resolution MALDI), the digest-matrix solution was spotted using the conventional dried-drop method and the spectra were externally calibrated with a standard peptide mixture. All measurements were made on a Reflex III mass spectrometer (Bruker Daltonics, Billerica, MA) operated in either positive ion linear mode (intact and CNBr digestion) or reflector mode (EndoLys-C digestion). A Voyager-DE MALDI-TOF-MS instrument (Applied Biosystems, Foster City, CA) operated in linear mode was also used for EndoLys-C digestion analysis.

2.9. In vitro and in vivo studies

To assess biological activity of the recombinant PA forms, a modification of a procedure based on the killing of macrophage cells by PA-LF toxin was used [58]. Two-fold serial dilutions of purified rPA63 or rPA83 (the latter expressed in *E. coli*) were incubated with 25 ng/mL rLF and 8×10^4 macrophage RAW264.7 cells/well in 96-well plates at 37 °C for 4 h. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (Promega)) and PMS (phenazine methosulfate, Promega) were added to final concentrations of 333 μg/mL and 25 μM, respectively, and incubation continued for 2 h at 37 °C. Cell viability was then determined by measuring the absorbance at 490 nm. Maximum macrophage viability was assessed in the absence of LF-PA. The ability of sera to neutralize toxin was performed by pre-incubation of a lethal concentration of the rPA83/rLF toxin (50 ng/mL rPA83 and 15 ng/mL rLF) with 2-fold serial dilutions of various serum samples (beginning at 1:100) for 1 h prior to adding the mixture to the cells. The assay was completed as described above. The EC_{50} (50% effective concentration) corresponds to the dilution of antiserum that resulted in 50% neutralization.

All animal procedures were conducted according to USDA regulations and were approved by both the Merck and USAMRIID Institutional Animal Care and Use Committees. New Zealand White rabbits, 1.5–3.5 kg, were obtained from Covance Research Products (Denver, PA). Juvenile male and female rhesus macaques, 1.5–2.5 kg, were obtained from the Merck NHP colony.

rPA63 was formulated for immunization by adsorption onto a proprietary Merck aluminum adjuvant (MAA) con-

Table 1
Schedule of immunizations, bleeds, and challenge

Species	No. of animals	Antigen	Immunizations (weeks)	Bleeds (weeks)	Challenge (weeks)
Rabbit	10	50 μg rPA63	0, 4, and 8	0, 2, 4, 6, 8, and 10	12
	10	5 μg rPA63	0, 4, and 8	0, 2, 4, 6, 8, and 10	12
	10	0.5 μg rPA63	0, 4, and 8	0, 2, 4, 6, 8, and 10	12
	10	MAA	0, 4, and 8	0, 2, 4, 6, 8, and 10	12
	5	Biothrax™	0, and 4	0, 2, 4, and 6	7
NHP	3	50 μg rPA63	0, 8, 24, and 38	0, 2, 4, 6, 8, 12, and 26	40
	3	5 μg rPA63	0, 8, 24, and 38	0, 2, 4, 6, 8, 12, and 26	40
	3	MAA	0, 8, 24, and 38	0, 2, 4, 6, 8, 12, and 26	40
	3	Biothrax™	0, 8, and 24	0, 2, 4, 6, 8, 12, and 26	40

taining 0.45 mg/mL aluminum. The protein was 0.2 μm-filtered, diluted with sterile saline to twice the desired final formulation concentration, and mixed with an equal volume of aluminum adjuvant. The formulation was mixed gently overnight at 4 °C and dispensed into sterile vials. Completeness of adsorption was determined by measuring the residual protein content of the supernatant fraction after pelleting of the adjuvant by centrifugation.

Rabbit and non-human primate challenge studies were conducted according to the immunization schedules shown in Table 1. Blood was collected at various time points and used for the determination of anti-PA titers by ELISA. Bacterial spores were administered via the inhalational route as described in previous publications [13,59,60]. Briefly, spores were suspended in sterile water to appropriate starting concentrations. The spores were then heat shocked for 45 min at 60 °C before aliquots were prepared for challenge. The non-human primates and rabbits were exposed in either a head-only, or nose-only exposure chamber, respectively. During the exposure, samples of the aerosol were collected in sterile water in an all-glass impinger. For each animal, the concentration of spores in the inhaled dose (expressed as LD_{50}) was determined by plating the collected samples onto tryptic soy agar plates.

Data from both rabbit and non-human primate challenge studies were analyzed using logistic regression [61]. The data were manipulated to allow concurrent analysis of both control and vaccine groups.

3. Results

3.1. Codon-optimized rPA63 expression in yeast

Analysis of the wild-type sequence for the gene encoding PA83 from *B. anthracis* Sterne strain (accession no. M22589 [62]) revealed that the codon usage of the bacterial gene differed significantly from that of genes known to express well in *S. cerevisiae*. Therefore, a synthetic PA63 gene sequence was constructed containing the same amino acid sequence encoded by the bacterial gene, but selected for yeast codons employed in highly expressed genes [63]. We evaluated both intra and extracellular expression of rPA63 in *S. cerevisiae* since it is difficult to predict a priori, which will be the most efficient means to produce a given heterologous protein. The synthetic PA63 gene was expressed under control of the inducible *GAL1* or *GAL10 Saccharomyces* promoters from constructs designed for either intracellular expression or secretion, respectively. Transformants were obtained in four different yeast strains containing *mnn9* and *prb1* mutations to eliminate hypermannosylation of N-linked glycosylation and reduce proteolysis, respectively [64]. Galactose induction of expression was achieved by an auto-induction protocol as detailed in Section 2 and production of rPA63 was determined by Western blot analysis.

We evaluated extracellular production first because *B. anthracis* PA is secreted, and we thought it had good potential to be secreted by *S. cerevisiae*. Small-scale evaluation of PA expression from numerous transformants (seven per each yeast strain) revealed that each produced an immunoreactive protein of ~75 kDa, which was 12-kDa larger than expected, based on comparison with tPA63 (data not shown). No cross-reactivity of the antibody was obtained in supernatants from numerous vector control transformants tested. In addition, expression levels of the immunoreactive protein were less than 1 μg/mL in the culture medium.

In contrast, a protein that co-migrated with tPA63 was obtained from a construct designed for intracellular expression in all 28 transformants tested (of four different yeast strains; data not shown). All of these transformants displayed volumetric titers of at least 5 μg/mL rPA63 in YEHDG culture medium. The best transformant, in terms of yield and quality of rPA63 produced, was strain 8-3, a transformant of strain 1260. Cultivation in complex YEHDG medium for 72 h at 30 °C was found to be optimal for expression. Fig. 1, lanes 7 and 8, show a Western blot depicting two different amounts of cell lysate from strain 8-3. A 63-kDa immunoreactive protein co-migrated with standard tPA63 that had been mixed with cell lysate prepared from a vector control transformant (lanes 1 and 2). The mixing was conducted to control for substances in the cell lysate that could potentially affect the quantity or quality of rPA63. It should be noted that no corresponding immunoreactive proteins were observed in cell lysate from a vector control transformant (data not shown). The absence of substantial low molecular weight products indicated that proteolytic processing of the intact

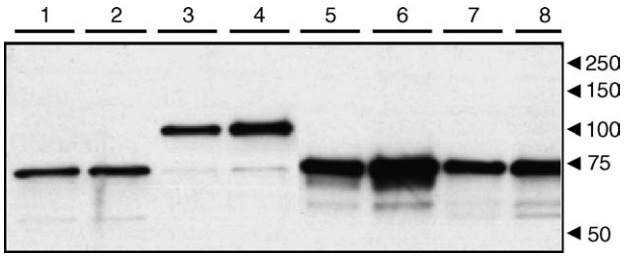

Fig. 1. Western blot depicting intracellular expression of rPA63 by strain 8-3. Lanes 1 and 2 represent 10 and 20 ng, respectively, of tPA63 mixed with 500 ng of total protein from the cell lysate of vector transformant 7-1. Lanes 3 and 4 represent 10 and 20 ng, respectively, of rPA83 (purified from *E. coli*), mixed with 500 ng protein cell lysate from vector transformant 7-1. Lanes 5 and 6 represent 250 and 500 ng, respectively, of cell lysate protein from 20-L fermentation (high density) of strain 8-3, while lanes 7 and 8 represent 250 and 500 ng, respectively, of cell lysate protein from small-scale cultivation of strain 8-3.

protein was minimal. The cultivation conditions described above resulted in a volumetric productivity of 65 and 80 μg rPA63/mL as determined by Western blot and ELISA, respectively, with rPA63 comprising 8% of the total yeast protein.

Fermentation of strain 8-3 was conducted in high-density medium. The major species produced co-migrated with tPA63 (Fig. 1, lanes 5, 6). The average concentration of rPA63 in the high-density fermentation, determined by both Western blot and ELISA, was 288 ± 97 μg/mL (avg. ± 1 SD), and rPA63 represented 10–12% of the total yeast protein as determined by densitometry of SDS polyacrylamide gels stained for total protein (as shown in Fig. 2A).

3.2. Purification of rPA63

Initial attempts to purify yeast rPA63 under non-denaturing conditions were unsuccessful. The rPA63 was not significantly enriched in any particular fractions using either anion or cation exchange chromatography. This behavior was observed for multiple column types and did not appear to be associated with a particular resin or manufacturer. Suspecting that multimerization of rPA63 might be the reason for the broad elution spectrum, we attempted to isolate the protein under denaturing conditions, where it would be largely unfolded and thus represent a single species.

rPA63 was successfully isolated under denaturing conditions from fermentor-grown cells cultivated in high-density medium. Clarified yeast lysate was adjusted to 6 M urea and processed by cation exchange chromatography. rPA63 eluted from the column as a sharp peak at approximately 100–150 mM sodium chloride. Fractions were pooled based on UV absorbance. The sodium chloride concentration of the pool was reduced by dilution with 6 M urea, and the pool was then applied to an anion exchange column. Fractions were again pooled based on UV absorbance, and dialyzed to remove denaturant. No protein precipitation was observed during dialysis. Subsequent sterile filtration of the dialyzed product pool did not result in any detectable loss of protein (recovery >99%). Purity of the final sterile-filtered pool was determined by densitometry and found to be greater than 90%. Recoveries of rPA63 for the major steps of the purification process are presented in Table 2. The yield of rPA63 through the purification process was approximately 2.5 g of rPA63/kg of cell paste, and the volumetric productivity of the

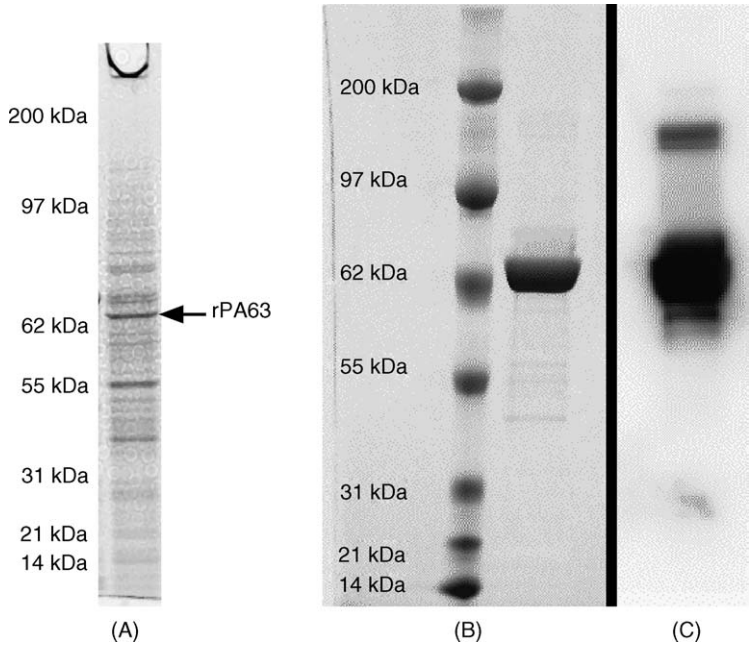

Fig. 2. SDS-PAGE analysis of the clarified yeast lysate from 20-L fermentation (A); and final product (B) of the purification process stained for total protein. (C) Western blot showing final product of purification.

Table 2
rPA63 recovery and purity at various steps in the purification

Sample	Volume (mL)	Protein (μg/mL)	Total Protein (mg)	Purity (%)
Clarified lysate[a]	320	13,200	4224	~17
Cation exchange pool	420	1000	420	~40
Anion exchange pool	240	589	141	>90

[a] The clarified lysate was prepared from a starting wet cell weight of 55.9 g.

fermentation was approximately 181 mg/L. Representative images of the results obtained by SDS-PAGE and Western blotting of the final product are shown in Fig. 2B and C.

3.3. Biochemical characterization of rPA63

A mass of 63,494 ± 11 Da was obtained for rPA63 as determined by MALDI-TOF-MS. This mass compares favorably with the predicted mass of 63,491. Endo Lys-c digestion produced a fragment at *m*/*z* 3175.9, which agrees with the rPA63 sequence of residues 1–30 (N-Ac-STSAGPTVPDRDNDGIPDSLEVEGYTVDVK) at an expected *m*/*z* (monoisotopic M + H) of 3175.5. This data confirms acetylation of the N-terminal serine, and there was no indication of non-acetylated peptide in the spectrum. The MALDI peaks for rPA63 were narrow and well defined, and mapping the protein with Endo Lys-C and CNBr digestions yielded 100% sequence coverage, confirming that none of the amino acids were modified. The CD analysis of yeast rPA63 and tPA63 is shown in Fig. 3A. The spectra of the two forms of PA63 are visibly different; with tPA63 showing a much more defined negative ellipticity near 220 nm, which would qualitatively suggest a higher alpha helical content. The minimum around 205 nm observed for rPA63 is suggestive of a more randomized structure. This observation is also consistent with the lower positive value of the ellipticity at 190 nm for rPA63 compared with tPA63. The spectra of rPA63 and tPA63 were analyzed by several programs (CDSSTR, SELCON3, and CONTINLL) used for estimation of secondary structure. The results of these analyses also suggest that yeast rPA63 has a higher degree of unordered structure than tPA63 (see inset in Fig. 3A). Fig. 3B shows the fluorescence spectra of rPA63 and tPA63 bound to the hydrophobic dye TNS. The higher observed intensity of yeast rPA63 at equivalent amounts of protein suggests that it has a higher hydrophobic character than tPA63.

3.4. Yeast produced rPA63 shows biological activity

The biological activity of yeast rPA63 was measured by its ability to combine with *E. coli* rLF and kill cultured macrophages. Fig. 4 shows that the purified yeast rPA63 was active in this assay, albeit at a higher concentration than control rPA83 purified from *E. coli*. *E. coli* rPA83 enabled 50% killing at a concentration of less than 10 ng/mL, while the rPA63 from yeast required approximately 1 μg/mL to achieve the same level of toxicity. The lower toxicity obtained with

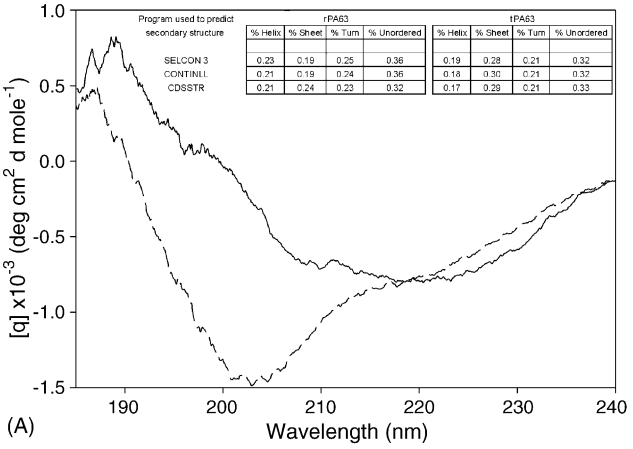

Fig. 3. (A) CD Spectra of rPA63 (dashed line) and tPA63 (solid line). (B) TNS fluorescence of rPA63 (dashed line), and tPA63 (solid line).

yeast rPA63 was not due to the presence of an inhibitor in the preparation as mixing experiments demonstrated that yeast rPA63 did not inhibit toxin activity when *E. coli* rPA83 and rLF were combined (data not shown). Neither yeast rPA63

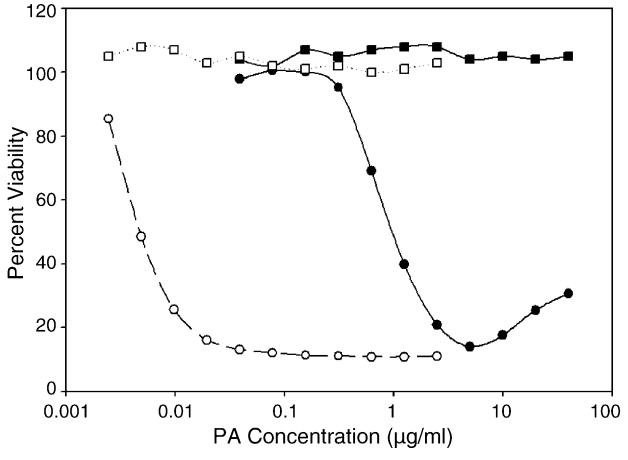

Fig. 4. Toxicity of rPA63 (closed circles) when combined with rLF in the macrophage-killing assay. *E. coli* produced rPA83 (open circles) was included as a control. The closed and open squares depict rPA63 and rPA83, respectively, in the absence of LF.

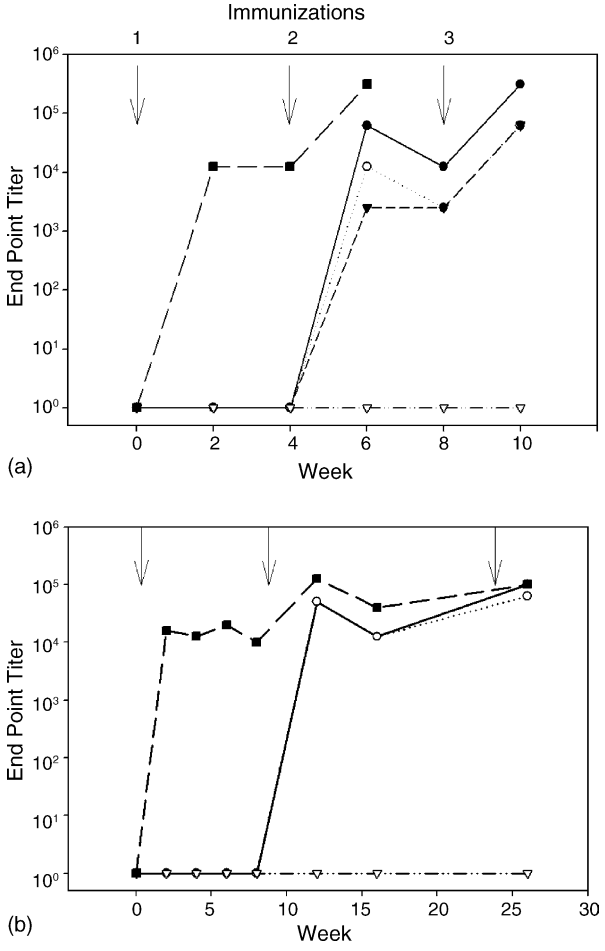

Fig. 5. (A) Time course showing rabbit end point titers to rPA83 following immunization with rPA63 (50 μg, closed circles; 5 μg, open circles; 0.5 μg, closed triangles), Biothrax™ (closed squares), or an adjuvant control (open triangles). (B) Time course showing NHP end point titers to rPA83 following immunization with rPA63 (50 μg, closed circles; 5 μg, open circles), Biothrax™ (closed squares), or an adjuvant control (open triangles). Immunizations are indicated by the inverted arrows in each plot. Note that the NHP 50 and 5 μg rPA63 titers overlap except for the final time point.

or *E. coli* rPA83 were observed to have any toxicity in the absence of rLF.

3.5. Purified rPA63 is immunogenic in rabbits and NHP

Rabbits and NHP immunized with the experimental yeast rPA63 vaccine mounted a vigorous response, producing IgG that was cross-reactive with rPA83 as shown in Fig. 5A and B, respectively. All rabbits and NHP in the test vaccine groups developed an immune response after two injections, and antibody titers continued to increase after the third injection. Animals receiving Biothrax™ showed increased titers after a single injection that were boosted after the second injection, and by a third injection that was given to NHP only. Rabbits receiving three 50 μg rPA63 doses reached equivalent titers to rabbits receiving two injections of one fourth of the human dose of Biothrax™ (Table 3a). NHP receiving

Table 3a
Geometric mean titers and survival of rabbits immunized with rPA63, Biothrax™, or MAA

Antigen	GMT[a]	Survival	LD_{50}[b]
50 μg rPA63	649,889	6 of 10	647 ± 216
5 μg rPA63	373,213	8 of 9	696 ± 184
0.5 μg rPA63	166,511	5 of 10	784 ± 268
Biothrax™	799,882	5 of 5	600 ± 107
MAA	1	0 of 10	651 ± 201

[a] Titers determined on blood collected 1 week prior to challenge.
[b] Value represents the average ± 1S.D.

Table 3b
Geometric mean titers and survival of rhesus macaques immunized with rPA63, Biothrax™, or MAA

Antigen	GMT[a]	Survival	LD_{50}[b]
50 μg rPA63	100,794	2 of 3	874 ± 692
5 μg rPA63	63,496	3 of 3	1350 ± 688
Biothrax™	100,794	2 of 3	795 ± 243
MAA	1	0 of 3	1226 ± 283

[a] Titers determined on blood collected 1 week prior to challenge.
[b] Value represents the average ± 1S.D.

three 50 μg doses of rPA63 had geometric mean titers similar to the NHP receiving three doses of Biothrax™ (Table 3b). The geometric mean titer for the 5 μg rPA63-immunized primates was approximately 50% of the Biothrax™ and 50 μg rPA63 groups.

3.6. Anti-rPA63 sera inhibits LF-PA-mediated killing of cultured macrophages

Individual antisera (collected 1 week prior to challenge) from rabbits that were immunized with the third 50 μg dose of yeast rPA63 (10 animals), the second dose of Biothrax™ (5 animals), and the third dose of MAA (2 animals) were tested for the ability to neutralize anthrax toxin in the macrophage killing assay. Two-fold serial dilutions from 100 to 102,400 were performed for each antiserum +/− LF-PA. None of

Table 4
EC_{50} determined in macrophage killing assay for sera from Biothrax™ and rPA63-immunized rabbits

Animal	Vaccine	No. of Immunizations	EC_{50}
1	Biothrax™	2	1672
2	Biothrax™	2	1521
3	Biothrax™	2	1484
4	Biothrax™	2	2511
5	Biothrax™	2	2652
1	rPA63	3	1075
2	rPA63	3	1282
3	rPA63	3	603
4	rPA63	3	1637
5	rPA63	3	996
6	rPA63	3	1573
7	rPA63	3	938
8	rPA63	3	2313
9	rPA63	3	2177
10	rPA63	3	1012

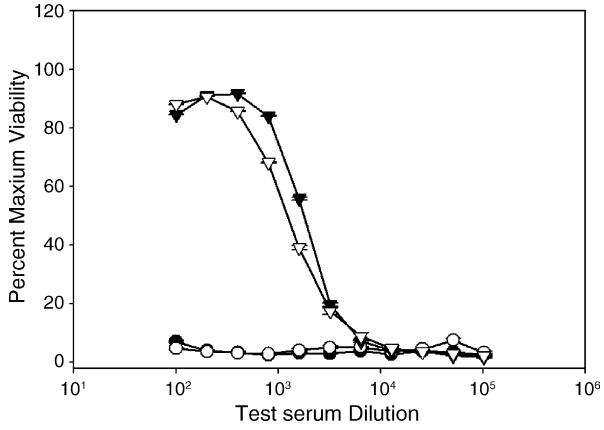

Fig. 6. Neutralization of PA-LF toxin by rabbit sera raised against rPA63 ($n = 10$, open triangles), and Biothrax™ ($n = 5$, closed triangles). Closed circles represent serum from pre-immune animals ($n = 3$) and open circles are the adjuvant control serum ($n = 2$). All values represent the average ± the standard error of the mean.

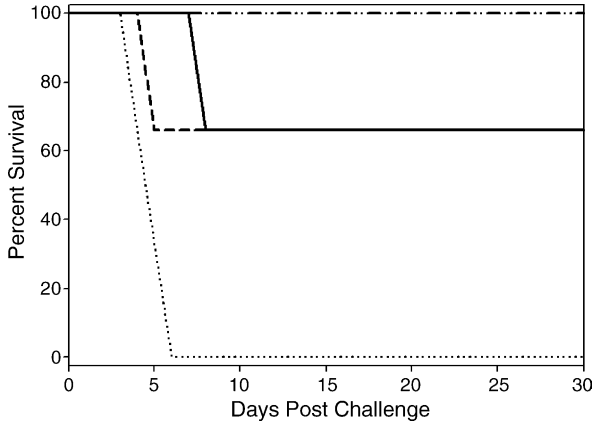

Fig. 7. Survival of NHP following an aerosol challenge with infectious anthrax spores. The lines are identified as follows: solid, Biothrax™; dotted, adjuvant control; dashed, 50 μg rPA63, and alternating dashed/dotted, 5 μg rPA63.

the antisera alone appeared to be toxic to the macrophages since no effect on viability was seen in the absence of LF-PA (data not shown). No inhibition of macrophage killing by LF-PA toxin was observed with MAA controls or pre-immune sera. Neutralization of toxin was obtained with antisera from all rabbits immunized with yeast rPA63 and Biothrax™ as shown in Fig. 6. The mean EC_{50} for the rPA63 group (Table 4) was approximately seventy percent that of the Biothrax group (1:1361 versus 1:1968, respectively), although this difference did not reach statistical significance (Mann–Whitney rank sum ($p = 0.076$)) [65].

3.7. rPA63 confers protection in two experimental models of inhalational infection

The rabbits in this study were challenged with approximately 675 LD_{50} spores of *B. anthracis* Ames strain at 4 weeks after the last injection of rPA63 or MAA, and 3 weeks after the last injection of Biothrax™. Survival of the rabbits receiving rPA63 or Biothrax™ was higher than the MAA controls as shown in Table 3a. All of the animals that received MAA died, whereas 19 of 29 rabbits receiving rPA63, and five of five receiving Biothrax™ survived. All doses of rPA63 showed equivalent protection ($p > 0.15$). Survival of rabbits in the immunized groups was significantly greater than the adjuvant control group ($p < 0.01$), and similar to survival in the group of rabbits given Biothrax™.

NHP were challenged with approximately 1000 LD_{50} spores of *B. anthracis* Ames strain. A survival plot for the non-human primate challenge is shown in Fig. 7. Survival of the monkeys vaccinated with rPA63 or Biothrax™ appeared significantly greater than the adjuvant-controls although there were not enough animals in the study to assign a *p*-value (Table 3b). The three control monkeys that received adjuvant alone died on days 4, 5, and 6 post-challenge. One of the three monkeys that received 50 μg rPA63 and one of the three that received Biothrax™ died on days 5 and 8 post-challenge, respectively. All of the remaining monkeys survived and showed no signs of illness as of day 21 post-challenge.

4. Discussion

This study was initiated after the 2001 anthrax attacks in order to determine the feasibility of producing a PA-based vaccine in *S. cerevisiae*. Our rationale for expressing the 63-kDa form of the antigen was: (a) interest in extracellular production which was thought to be more achievable with the mature protein; (b) concern that intact PA83 would be difficult to isolate due to endogenous yeast protease activity; (c) literature reports suggesting that protective epitopes were present on PA63 [1,22–24]; and (d) the potential that PA63 might contain additional protective epitopes generated by conformational changes resulting from protease cleavage of the 83-kDa protein. Indeed both PA63 monomer and PA63 heptamer/LF complexes have been detected in the blood of infected animals, whereas PA83 has not [66,67]. *S. cerevisiae* was chosen based on in-house experience with this expression system for vaccine manufacturing, and because of the excellent safety profile of currently licensed and experimental yeast-derived vaccines such as Recombivax™ [68,69], and Gardasil™ [38].

The current state of understanding of protein production in yeast does not allow one to predict which proteins will be made in sufficient quantities for commercial production. The degree of glycosylation, extent of protein degradation, and correct polypeptide folding are all variables that must be determined empirically for a particular recombinant protein.

Because PA is a protein that is secreted naturally by *B. anthracis*, we first tried to express rPA63 as a secreted extracellular protein in *S. cerevisiae* to facilitate downstream purification. Several difficulties were encountered in this line of experimentation. PA63 produced via the secretory pathway

migrated in SDS-PAGE gels at an apparent molecular weight of 75 kDa, which is approximately 12,000 Da higher than the predicted molecular weight for the activated form. The increase in mass could have resulted from N-glycosylation since rPA63 contains eight potential Asn-X-Ser/Thr consensus sequences. Although the rPA63 construct was expressed in *mnn9* mutants to prevent elongation of N-linked glycosylation, these mutations do not preclude core glycosylation. There are also numerous potential sites for O-glycosylation, which is even more difficult to predict. Alternatively, the higher molecular weight could be due to improper cleavage of the alpha factor pre-pro leader sequence, which was used to direct the protein to the secretory pathway. Expression of proteins in *S. cerevisiae pep4* mutants sometimes improves cleavage of lys-arg-containing leader sequences (like alpha factor) by Kex2 protease, since Kex2p is a substrate of Pep4 [40]. However, the molecular weight of rPA secreted in *pep4* mutants was the same as in strains that contained wild-type *PEP4* (data not shown). The alpha factor pre-pro leader amino acid sequence in vector pKH4α2 differs from the authentic leader by two amino acids due to the introduction of a convenient cloning site [44]. Expression of rPA63 in a different secretory vector, pKH4B [46], containing an authentic alpha-factor pre-pro leader, did not improve either the quality or quantity of rPA63 secreted (data not shown). A major disadvantage to extracellular expression was that very low volumetric productivity was obtained under numerous fermentation conditions, despite the fact that the gene sequence was codon-optimized for *S. cerevisiae*. Future studies are needed to discern whether rPA63 is indeed glycosylated and/or contains uncleaved leader sequence in order to optimize secretion of rPA63 in yeast.

In contrast to our attempts to secrete rPA63, we were successful in achieving high-level expression of rPA63 intracellularly. The highest specific productivity was obtained in a protease and glycosylation-deficient strain enhanced for over-expression of the *Saccharomyces GAL4* transcription factor. Intracellular production of rPA63 scaled-up favorably from shake-flasks to fermentors in terms of both specific content, and percent of total protein. There was no evidence of significant proteolytic degradation of rPA63 in either small-scale cultivations (tube or flask), or 20-L fermentations.

Purification of rPA63 provided an unanticipated challenge. Isolation of the protein under native conditions was not achieved. A variety of anion and cation exchange matrices were evaluated under non-denaturing conditions, and in each case the product bled slowly off of the column instead of eluting as a defined peak. A possible explanation for this behavior rests with the oligomerization-prone nature of PA63. In vivo, receptor-bound PA63 self-associates to form heptamers capable of binding LF and/or EF. In yeast, the structural characteristics of the protein responsible for self-association may result in production of multiple oligomeric forms of PA, or formation of PA—yeast protein complexes intracellularly or during early stages of the purification process, thus, preventing efficient isolation under native conditions.

rPA63 elutes as a well-defined peak when isolated under denaturing conditions using ion exchange chromatography in the presence of urea. The purified product showed minimal proteolysis by SDS-PAGE and Western blot analyses. We were concerned that urea-induced carbamylation may occur during the purification process. In order to minimize the potential for carbamylation, all steps of the process were performed at 2–8 °C using freshly prepared buffers. The ion-exchange based chromatographic separations precluded the use of guanidine hydrochloride as a denaturant. The precautions were successful, as no evidence of carbamylation was identified by MALDI-MS of the purified product. The only modification observed by MALDI-MS was acetylation of the N-terminal serine residue, a co-translational modification that commonly occurs in recombinantly expressed proteins [70]. The yield of rPA63 through the purification process was approximately 2.5 g of rPA63/kg of cell paste (∼181 mg/L), which compares favorably with the recovery of rPA83 from *E. coli* [34,35], and from a nonsporogenic, avirulent strain of *B. anthracis* [71].

Several lines of evidence indicated that the rPA63 protein isolated under denaturing conditions was not completely refolded to a "native" state: (1) The CD spectra of rPA63 and tPA63 are visibly different, with the latter exhibiting a qualitatively higher alpha helical content. Secondary structural analysis of the spectra also suggest that yeast rPA63 has a higher degree of unordered structure than tPA63, however, a limitation of many such analysis programs is that they rely on data from a set of reference proteins that may or may not be valid for the evaluation of the protein of interest. (2) TNS-binding fluorescence intensity was higher for yeast rPA63 compared to tPA63. As TNS fluorescence is related to the hydrophobic character of a protein [72], this data indicates that exposure of hydrophobic amino acid residues was more pronounced in the yeast-derived product. Sequestration of hydrophobic regions is one of the primary entropic-driven forces directing protein folding, and therefore, differences in the solvent-exposed hydrophobicity of constructs with identical primary sequences is strongly indicative of differences in secondary structure. (3) Finally, rPA63 was clearly less effective at facilitating LF toxicity than an equivalent amount of *E. coli* rPA83 in the macrophage killing assay [72], although both rPA63 and rPA83 showed a dose-dependent activity. Yeast expressed rPA63 may not be fully active for a number of reasons related to improper refolding of the protein upon renaturation. These would include defective heptamerization, impaired binding to the cell-surface anthrax toxin receptor, impaired binding of the heptamerized PA to LF or EF, or impaired pore formation.

Despite the fact that yeast-expressed rPA63 was sub-optimally folded, it was highly immunogenic in rabbits and non-human primates after two injections. Furthermore, it provided protection from an infectious challenge that compared favorably with Biothrax™ in both the rabbit and non-human primate models of inhalational infection. A direct comparison of yeast-expressed rPA63 and Biothrax™ could not be

made in the NHP model as the time between the final boosts and challenge differed due to an unanticipated scheduling conflict after the experiment had been initiated. In rabbits, all doses of rPA63 (50, 5, and 0.5 μg) and Biothrax™ showed equivalent protection, and survival in all immunized groups was significantly greater than in the adjuvant control group. The protection obtained in the NHPs is noteworthy as they were exposed to a spore challenge dose that was five times greater than the established LD_{50} for this species. Nevertheless, five of six animals immunized with either 5 or 50 μg of rPA63 survived, as did two out of the three NHP that received Biothrax™. Statistical analysis of the primate data was not warranted due to the small sample sizes involved, however, the ability of rPA63 to protect against anthrax infection does compare favorably with a prior study demonstrating protection by rPA83 in rhesus macaques [73]. The data are also in agreement with a communication describing protection against challenge with infectious spores of the *B. anthracis* Sterne strain by immunization of guinea pigs with tPA63 [25].

The question of whether rPA63 has any advantages relative to rPA83 as a vaccine antigen was beyond the scope of this study and is a topic for future study. However, rPA63 seems advantageous for expression and purification in yeast. We did express codon-optimized rPA83 intracellularly in *S. cerevisiae* and found that higher yields were obtained with rPA63, and the rPA83 tended to be more sensitive to the presence of yeast proteases (data not shown).

Hermanson et al. found that a cationic lipid-formulated rPA83 (lacking the furin cleavage site) plasmid DNA vaccine was more efficacious than a similar rPA63 construct with respect to immunogenicity in mice and the ability of mouse antisera to neutralize Letx [74]. The rPA83 construct was further evaluated and shown to protect rabbits against inhalational challenge with spores from the Ames strain. Greater survival of the rabbits was obtained by immunization with the rPA83 construct than with our rPA63.

This work supports the results of previous studies by Ramirez et al. [71], which demonstrated that recombinant PA83 altered to prevent protease activation and heptamerization did generate high levels of IgG with toxin-neutralizing activity. However, our study is the first to demonstrate that a completely functional PA is not required for protection against anthrax infection in a NHP inhalational challenge model. An inactive form of rPA83 referred to as DNI was highly immunogenic and conferred protection against anthrax lethal toxin in a mouse model [75]. Whether this antigen or the rPA83 construct that lacks the furin cleavage site and is presumably biologically inactive, are also protective in a NHP inhalational challenge model remains to be demonstrated.

In conclusion, the 63-kDa form of *B. anthracis* PA was expressed in the yeast *S. cerevisiae* following codon optimization of the bacterial PA63 sequence. rPA63 was expressed at high levels in the yeast cells, and purification of the protein under denaturing conditions resulted in a preparation that was greater than 90% pure following two chromatographic steps. The rPA63 produced in this manner had biological activity as evidenced by its ability to combine with lethal factor and kill cultured macrophages in vitro, despite biophysical evidence for sub-optimal refolding. This data strongly suggests that proper refolding of rPA63 might not be essential for protection as evidenced by the ability of sera from immunized animals to prevent killing of cultured macrophages. More importantly, immunization of rabbits and non-human primates with rPA63 resulted in protection from lethal challenge at levels comparable to those found with the currently licensed anthrax vaccine.

Acknowledgements

This work was funded by Merck and Company. Animal studies were performed in collaboration with the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) under a Collaborator Research and Development Agreement (CRDA; number DAMD17-02-0175). We thank Cameron Douglas and Randy Greasham for helpful discussions and reagents. We are grateful to Patricia Cameron for providing *E. coli*-expressed rLF, and to Charles Tan for statistical analyses of the rabbit and NHP challenge studies.

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